APPENDIX A

Antisense RNA Amplification using a modification of the method described in Van Gelder *et al.* U.S. Patent No. 5,716,785

A. Reagents:

First Strand cDNA Synthesis

1. Poly-A RNA. Human HeLa Cell poly-A RNA can be purchased from Clontech, P/N 6522-1
2. DNA T7T18VN (20 μM): (5') AAT TAA TAC GAC TCA CTA TAG GGA GAT TTT TTT TTT TTT TTT TTV N (3') (V = A/C/G, N = A/C/G/T)
3. Superscript II Reverse Transcriptase, Life Technologies P/N 18064-014
4. RNAGuard, Pharmacia P/N 27-0815-01
5. 5x First Strand Buffer*: 250 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 375 mM KCl
   * supplied with Superscript II Reverse Transcriptase, Life Technologies
6. 100 mM DTT*
   * supplied with Superscript II Reverse Transcriptase, Life Technologies
7. dNTPs (10 mM each), diluted from Pharmacia P/N 2702035-01
8. Nuclease-free water, Amresco P/N E476

Second Strand cDNA Synthesis

12. *E. coli* DNA polymerase I, Life Technologies P/N 18010-017
12. *E. coli* RNaseH, Life Technologies P/N 18021-014
12. *E. coli* DNA Ligase, Life Technologies P/N 18052-019
12. T4 DNA polymerase, Epicentre P/N D0602H
12. 5x Second Strand Buffer: 100 mM Tris-HCl, pH 6.9, 23 mM $MgCl_2$, 450 mM KCl, 0.75 mM β-NAD^+, 50 mM $(NH_4)_2SO_4$, Life Technologies P/N 10812014
12. dNTPs (10 mM each), diluted from Pharmacia P/N 2702035-01
12. Nuclease-free water, Amresco P/N E476
12. EDTA 0.5 M, Amresco P/N E522 (or equivalent)
12. Phenol:chloroform:isoamyl alcohol (25:24:1), Amresco P/N 0883 (or equivalent)
12. Ammonium Acetate 7.5 M, Sigma P/N A2706
12. 100% ethanol, Amresco P/N E193
12. 70% ethanol, diluted from Amresco P/N E193

Transcription Reaction

1. T7 RNA Polymerase, Epicentre P/N TU950K
2. RNAGuard, Pharmacia P/N 27-0815-01
3. Inorganic Pyrophosphatase (200 U/ml)
4. 5x Transcription Buffer: 0.2 M Tris-HCl, pH 7.5, 50 mM NaCl, 30 mM $MgCl_2$, 10 mM spermidine, Epicentre P/N BP001
5. 100 mM DTT, Epicentre P/N BP001
6. $MgCl_2$ (400 mM), diluted from Sigma P/N M-1028
7. NTPs (25 mM each), diluted from Pharmacia P/N 27-2025-01
8. Nuclease-free water, Amresco P/N E476
9. Lithium Chloride (4.0 M), diluted from Sigma P/N L-7026
10. 70% ethanol, diluted from Amresco P/N E193
11. TE pH 8.0, Amresco P/N E112

B. Procedure:

1. Add 100 ng to 600 ng poly-A RNA to reaction tube. Add 1.0 µl DNA T7T18VN (20 µM) and bring total sample volume to 11.5 µl in nuclease-free water. Incubate 70 C for 10 min to denature primer and template. Quick chill on ice.

2. Mix the following components and maintain on ice. For more than one reaction, multiply by the number of reactions.

Master Mix A	
Component	Volume (µl)
5x First Strand Buffer	4.0
100 mM DTT	2.0
dNTPs (10 mM each)	1.0
Superscript II RT (200 U/µl)	1.0
RNAGuard (36 U/µl)	0.5
Volume of Master Mix A	8.5

3. Aliquot 8.5 µl of the master mix A into each sample tube. Incubate first strand synthesis reaction at 37 C for 60 min.

Composition of First Strand Synthesis Reaction	
Component	Final concentration or amount
poly-A RNA	600 ng
DNA T7T18VN	1 µM
Tris-HCl, pH 8.3	50 mM
$MgCl_2$	3 mM
KCl	75 mM
DTT	10 mM
dNTPs	0.5 mM each
Superscript II RT	200 U
RNAGuard	18 U
Total reaction volume	20 µl

4. Mix the following components and maintain on ice. For more than one reaction, multiply by the number of reactions.
Note: If the activities of the enzymes are different than indicated below, add the appropriate volume to give the Units per reaction indicated below.

Master Mix B		
Component	Volume (µl)	Units
Nuclease-free water	91	
5x Second Strand Buffer	30	
dNTPs (10 mM each)	3.0	
E. coli DNA Ligase (10 U/µl)	1.0	10
E. coli DNA polymerase I (10 U/µl)	4.0	40
E. coli RNaseH (2 U/µl)	1.0	2
Volume of master mix B	130	

5. On ice, aliquot 130 µl of the Master Mix B into each sample. Incubate second strand synthesis reaction at 16 C for 120 min.

Note: Second strand synthesis reactions are incubated at 16 C to inhibit strand displacement by DNA polymerase I. Do not let the temperature rise above 16 C.

Composition of Second Strand Synthesis Reaction	
Component	Final concentration or amount
Single-strand cDNA	Approximately 600 ng
Tris-HCl, pH 7.5	25 mM
$MgCl_2$	5 mM
KCl	100 mM
β-NAD^+	0.15 mM
10 mM $(NH_4)_2SO_4$	10 mM
DTT	1.2 mM
dNTPs	0.25 mM each
E. coli DNA Ligase	10 U
E. coli DNA Polymerase I	40 U
E. coli RNaseH	2 U
Total reaction volume	150 µl

6. Add 2 µl T4 DNA polymerase (10 U) to each sample tube. Incubate 16 C for 5 min.

7. Add 10 µl of 0.5 M EDTA to each sample tube.

8. Add 150 µl of phenol:chloroform:isoamyl alcohol (25:24:1). Vortex thoroughly and spin 5 min at room temperature in microcentrifuge. Carefully remove 140 µl of the upper, aqueous phase and transfer to fresh reaction tube.

9. Add 70 µl 7.5 M NH_4OAc and 0.5 ml 100% ethanol to each sample tube. Mix and spin 20 min at 4 C in microcentrifuge. Rinse each sample pellet in 70% ethanol. Dry pellet briefly at room temperature. Resuspend each sample pellet in 40 µl nuclease-free water.

10. Immediately before use, mix the following components at room temperature. For more than one reaction, multiply by the number of reactions.

Master Mix C	
Component	Volume (µl)
Nuclease-free water	4.1
5x Transcription Buffer	16
100 mM DTT	8.0
NTPs (25 mM each)	8.0
400 mM $MgCl_2$	2.0
RNAGuard (36 U/µl)	0.5
Inorganic Pyrophosphatase (200 U/µl)	0.6

T7 RNA polymerase (2500 U/μl)	0.8
Volume of Master Mix C	40

11. Aliquot 40 μl of the master mix C into each sample tube. Incubate transcription reactions at 37 C for 60 min.

Composition of Transcription Reaction	
Component	Final concentration or amount
Double-strand cDNA	Approximately 1200 ng
Tris-HCl, pH 7.5	50 mM
$MgCl_2$	16 mM
NaCl	10 mM
Spermidine	2 mM
DTT	10 mM
NTPs	2.5 mM each
T7 RNA polymerase	2000 U
RNAGuard	18 U
Inorganic pyrophosphatase	0.12 U
Total reaction volume	80 μl

12. Add 20 μl 50 mM EDTA to each reaction tube to stop the reaction. Alternatively, if quantitation of the amplified RNA product is desired, purifiy the RNA from unincorporated nucleotides by precipitation of the antisense RNA products in 2.0 M LiCl. Measure the RNA concentration by absorbance at OD_{260} using the conversion: 1 OD_{260} = 40 μg/ml RNA.